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(57) Abstract

Animals bearing an immortalizing gene together with one or more genes of interest, and cell lines capable of long term growth *in vitro* are described. Cell lines derived from F1 Immorto/Min mouse hybrid carry a defective Apc allele and are conditionally immortalized by virtue of an expression of a temperature sensitive SV40 large T antigen. These cell lines may further be transfected with other genes of interest such as the Ras oncogene to render them tumourigenic.

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NOVEL ANIMALS AND CELL LINES

This invention relates to animals bearing an immortalizing gene together with a gene of interest, and cell lines capable of long term growth *in vitro*. In a preferred embodiment, the invention relates to conditionally immortalized cell lines derived from mice carrying the Multiple Intestinal Neoplasia gene, which predisposes to colon cancer, and a temperature sensitive mutant of the SV40 large T gene which allows growth at certain temperatures. Conditionally immortalized cell lines have been established from small intestinal epithelium, colonic mucosal epithelium, liver epithelium and stomach epithelium. Fibroblast cell lines from small intestinal stroma, colon, muscle and skin have also been established. These animals and cell lines are useful in studies of aberrations of growth and differentiation, including carcinogenesis that may be induced, for example, by viruses, various genes or mutagens.

Background and Prior Art

Experimental systems which can be used to study the factors involved in transformation and carcinogenesis have been available for many years, but most of these systems either require the use of carcinogenic chemicals, or rely on vertically-transmitted viruses, experimental virus infection, or other factors which render the experimental system poorly analogous to cancer in humans. Recently it has been discovered that many human cancers involve a genetic predisposition. In particular, some of the commonest solid tumours have now been found to be associated with specific genes, and in some cases, even where there is no familial predisposition, a particular gene mutation has been identified. For example, genes have been identified which predispose to breast cancer and to colonic cancer. In some cases, analogues of these genes in other mammalian species have been identified. Furthermore, many animal oncogenes are known, particularly in mice.

Bowel cancer, and in particular colonic cancer, is one of the most common solid tumours to affect humans. Familial Adenomatous Polyposis is a genetically-determined condition resulting from a mutation of the Adenomatous Polyposis Coli (APC) gene. Individuals with Familial Adenomatous Polyposis have a greatly increased propensity to develop colon cancer, and mutations of the APC gene are also found in patients with non-familial (ie. sporadic) colon cancer.

With the development of the Multiple Intestinal Neoplasia (Min) mouse (1), a mouse model of Familial Adenomatous Polyposis in humans became available (2-4). The usefulness of this model was enhanced by the finding that the Min mutation is a nonsense mutation in codon 15 of the mouse APC gene (5). This gene is the mouse homologue of the human APC gene (3) which is mutated in most Familial Adenomatous Polyposis patients (6,7). The Min mouse has been shown to carry a dominant mutation of the murine homologue of the human APC gene, which expresses its phenotype in all mice bearing the mutant allele as multiple intestinal tumours, in both small intestine and colon, at an early age, usually by 120 days (1).

Mutations of the APC gene comprise one of a series of mutations and deletions in different genes that occur during the development of both familial and sporadic colon cancer in humans (6,7,18). The APC gene appears to become mutated early in the sequence from normal colonic epithelium to adenoma to malignancy that occurs in colon cancer (18). A second gene, the tumour suppressor gene Deleted in Colon Carcinoma (DCC), which is a gene mutated in both alleles of chromosome 18q, is present in approximately 70% of human colon carcinomas. The protein encoded by this gene is similar in structure to members of the cellular adhesion molecule (CAM) group of proteins, which are known to be involved in processes of development and differentiation. Another tumour suppressor gene, the p53 gene in chromosome 17, is also an important contributor

to the oncogenic phenotype in colon cancer. Murine analogues of these genes have been at least partially cloned and characterised.

The usefulness of the Min model system has been severely limited because of the difficulty of culturing intestinal epithelial cells, both from the colon and from the small intestine, in long term culture (8-10). Thus, so far, only *in vivo* studies have been possible because of the lack of an *in vitro* model system.

Epithelial cell lines from both the small intestine and colon of adult mice (11) using an unique transgenic mouse, the "Immortomouse" (H-$2K^b$-tsA58 SV40 large T transgenic mouse), which carries a temperature sensitive (ts) mutant of the SV40 large T gene (12) were recently established. These cells only grow at the permissive temperature (33°C), indicating that they are conditionally immortalized. Growth of the cells requires the presence of γ-interferon because of the presence of a γ-interferon inducible element in the H2Kb promoter incorporated with the SV40 large T gene (13). The intestinal epithelial cell lines expressed known brush border-associated enzymes (11).

The Immortomouse strain has now been used to establish F1 Min/Immortomouse hybrids which carry both the SV40 large T gene and the Min mutation. Described herein is the establishment of a conditionally immortalized epithelial cell line from the colonic mucosa of an F1 hybrid mouse which carries the Min allele. It was necessary to use a hybrid strain carrying an immortalizing gene, as it was not possible to culture the normal colonic epithelial cells of adult animals for more than a few days (8,9,19). Conditionally immortalized Min/+ cell lines have also been derived from the liver and small intestinal stroma of the same mouse, and other conditionally immortalized Min/+ cell lines have been established.

All these lines carry the Min mutation and are useful in studies investigating the apparent tissue

specificity of the action of mutations in the formation of tumours in the Min mouse model.

The Immortomouse is useful for developing cultures from tissues, such as the intestine, that have previously proved very difficult to culture *in vitro*. Thus the invention provides a generally-applicable method for obtaining cell lines of a desired mammalian tissue carrying one or more genes of interest. Using the same principle, a cell line lacking a gene of interest can be obtained. An animal either carrying or deficient in a gene of interest is crossed with an animal of the same species carrying an immortalizing gene in its germ line. Thus the Immortomouse can be crossed with any other transgenic mouse strain carrying a gene of interest, to obtain progeny expressing both the SV40 large T gene and the gene of interest. For example, the Immortomouse can be crossed with a mouse bearing the Ras oncogene. Alternatively, the Immortomouse can be crossed with a mouse in which a gene of interest has been specifically disrupted, to obtain immortalized cells in which the gene of interest is not expressed. For example the Immortomouse can be crossed with a mouse in which a gene for a growth factor such as transforming growth factor α, fibroblast growth factor, or epidermal growth factor is disrupted. Organs from these mice will yield tissue that can be cultured *in vitro*.

The animals and cell lines of the invention are especially useful in studies such as:

a) detection of tissue specific markers;
b) effects of growth factors on cells of different tissues;
c) effects of oncogenes associated with cancers of different tissues;
d) screening of putative initiators, promoters and inhibitors of malignant progression;
e) screening of putative therapeutic agents;
f) screening of putative growth factors;

g) the effect of the gene(s) of interest or its absence on differentiation, senescence and cell death in tissues which undergo these processes;

h) effect of possible modifiers on differentiation, senescence and cell death, in tissues bearing or lacking the gene of interest; and

i) cell-cell interactions between cells of different lineages.

Summary of the Invention

In one aspect, the invention provides an F1 hybrid non-human animal, of which one parent carries an immortalizing gene, and the other parent carries one or more genes of interest.

The immortalizing gene may be any gene which confers the capacity for long-term growth in cell culture. Suitable immortalizing genes include, but are not limited to, the SV40 large T gene, the adenovirus E1A gene, and the polyoma virus middle T gene. Combinations of immortalizing genes may be used. Other suitable genes will be known to those skilled in the art. The only requirement is that the immortalizing gene is present in the germ line of one of the parental strains, and consequently is also present in all somatic cells of that parental strain. Preferably the animal is a mammal, preferably a rodent, especially a rabbit, rat or mouse.

The gene of interest may be any gene for which a transgenic animal of the appropriate species can be generated, and is preferably one which confers a predisposition towards development of abnormalities such as cancer. Thus the gene of interest is preferably a cancer-associated gene, an oncogene, a tumour suppressor gene, or a tumour inhibitor gene. The gene may bear one or more mutations, and/or it may be a transgene, including a transgene of human origin. Preferably the gene of interest is selected from the group consisting of Multiple Intestinal Neoplasia (Min) and its analogues, Adenomatous

Polyposis Coli (APC), Deleted in Colon Carcinoma (DCC), or from the group consisting of Ras, Myb, Myc, Raf, p53 and p16. In a particularly preferred embodiment, the gene of interest is the Multiple Intestinal Neoplasia gene, or its homologue in other animals. In another particularly preferred embodiment, the animal carries both a cancer-associated gene and an oncogene.

Alternatively, the gene of interest may be any gene for which a disruption of expression can be generated and transmitted via the germ line in a so-called "knockout" animal. Many "knockout" animals are now known, particularly knockout mice, including many mouse strains in which growth factors or cytokines are not expressed. Thus where the gene of interest is disrupted, the gene is preferably one encoding a cytokine, growth factor, hormone or enzyme. Preferred such cytokines and growth factors include, but are not limited to, interleukins, interferons, epidermal growth factor, fibroblast growth factor, and transforming growth factor, particularly transforming growth factor α. For the purposes of this specification, an animal in which the expression of a gene of interest is selectively disrupted will be referred to as an animal lacking that gene of interest.

In a preferred embodiment, the invention provides a F1 hybrid animal, of which one parent carries a temperature-sensitive mutant of the SV40 large T gene, and the other parent carries or lacks a gene of interest.

Preferably the parental strain carrying the temperature-sensitive mutant of the SV40 large T gene is a rodent, for example the Immortomouse (H-2K^b-tsA58 SV40 large T).

According to a second aspect, the invention provides an immortalized or conditionally immortalized cell line derived from an animal according to the invention. The cell line may be derived from any tissue of the animal which is capable of growth in cell culture, and epithelial cells and fibroblasts are especially suitable.

Preferably the cell line is selected from the group consisting of epithelial cells such as colonic epithelial cells, liver epithelial cells, small intestinal epithelial cells, and fibroblasts such as small intestinal fibroblasts, muscle fibroblasts and skin fibroblasts. The cell line may be transfected with further genes, for example, the Ras oncogene.

Preferably the cells of these cell lines carry both the SV40 large T gene and one or more genes of interest. Preferably the gene of interest is selected from the group consisting of Multiple Intestinal Neoplasia (Min), Adenomatous Polyposis Coli (APC), Deleted in Colon Carcinoma (DCC), Ras, Myb, Myc, Raf, p53 and p16. In a particularly preferred embodiment, the gene of interest is the Multiple Intestinal Neoplasia gene. In a more preferred embodiment, the genes of interest are the Multiple Intestinal Neoplasia gene and the Ras oncogene.

According to a third aspect, the invention provides a method of obtaining an immortalized or conditionally-immortalized cell line carrying or lacking one or more genes of interest, comprising the steps of crossing a non-human animal bearing an immortalizing gene with an animal of the same species bearing or lacking the gene of interest to obtain an F1 hybrid animal, and culturing cells from the F1 hybrid animal under conditions suitable for growth thereof.

In a particularly preferred embodiment this aspect of the invention provides a method of obtaining a conditionally-immortalized cell line carrying or lacking one or more genes of interest, comprising the steps of crossing a mouse carrying or lacking the gene(s) of interest with a mouse carrying a temperature sensitive mutant of the SV40 large T gene to obtain an F1 hybrid mouse, and culturing cells from the F1 hybrid mouse at the permissive temperature.

According to a fourth aspect, the invention provides a method of screening agents suspected of inducing

or predisposing to development of cancer, such as mutagens, oncogenes, carcinogens, co-carcinogens, viruses and growth factors, or of agents suspected to be useful in treatment or prevention of cancer, such as therapeutic agents, immunological response modifiers, receptors, hormones, antibodies, cytokines, and growth factors, comprising the step of exposing an animal or cell line of the invention to the agent to be tested. This aspect of the invention also includes within its scope the use of cell lines of the invention derived from different tissues of the same animal to identify the role of tissue-specific elements in the response to such agents. It further includes the use of the cell lines to identify novel oncogenic partners which co-operatively induce transformation, their use to identify genetic changes occurring as a result of experimental manipulation of the cell, and to their use in screening novel, cancer-causing genes using DNA from tumours or tumour homogenates. The tumours targeted are preferably solid tumours such as colon, breast, bladder or ovarian tumours. Tissues from a tissue bank or from a fresh surgical sample may be used.

While the invention is specifically described with reference to the Immortomouse/Min hybrid, it will be clearly understood that this merely illustrates the broad principle of the invention, which is generally applicable as set out above.

Detailed Description of the Invention

The invention will now be described in detail by way of reference only to the following non-limiting examples, and to the drawings, in which:

Figure 1 shows a phase contrast micrograph of colonic epithelial cells cultured from crypts isolated from the colonic mucosa of a young adult Immortomouse/Min hybrid mouse (Magnification is x125);

Figure 2 illustrates staining of colonic epithelial cells at passage 6 with a monoclonal antibody

specific for keratin 18 (LE61) (Magnification is x600);

Figure 3 shows a phase contrast micrograph of liver epithelial cells cultured from the liver of the Immortomouse/Min liver (Magnification is x125);

Figure 4 shows the results of an assay demonstrating the conditional immortalisation of the colonic epithelial cells. The cells were cultured at either the permissive temperature (33°C) or the non-permissive temperature (39.5°C) for 6 days, both with and without mouse γ-interferon (IFN; 10 units per ml). The cell numbers in each well were counted. The results shown are the means of triplicate cultures;

Figure 5 shows the PCR products of cell lines derived from an Immortomouse/Min hybrid mouse.

Lanes marked 1 to 7:	reactions using the SV40 large T primers;
Lanes 8 to 14:	PCR products derived from the Min oligonucleotides;
Lanes 1 and 8:	negative controls with no DNA;
Lanes 2 and 9:	derived from DNA prepared from mice which carried neither SV40 large T nor the mutated Min allele;
Lanes 3 and 10:	positive controls from Immorto/Min mice.
Lanes 7 and 14:	Young adult mouse colon (YAMC) cell lines which contain the SV40 large T gene but not the Min gene.
Lanes 4 and 11:	DNAs from the colonic epithelial cell line from the Immorto/Min mouse cell lines
Lanes 5 and 12:	fibroblasts derived from the small intestine; and
Lanes 6 and 13:	liver epithelium.

Figure 6 shows the results of analysis of YAMC-Ras and IMCE-Ras colonic cell lines for protein expression and genotyping.

Figure 7 shows the analysis of growth kinetics of YAMC-Ras and IMCE-Ras cell lines under a) permissive and b) non-permissive culture conditions.

Figure 8a shows the morphology of cell lines: i) YAMC cells, ii) YAMC-Ras cells, iii) IMCE cells, and iv) IMCE-Ras cells;

Figure 8b shows the growth of cell lines in soft agar: i) YAMC cells, ii) YAMC-Ras cells grown under permissive culture conditions, iii) YAMC-Ras cells grown under non-permissive culture conditions, iv) IMCE cells, v) IMCE-Ras cells grown under permissive culture conditions, and vi) IMCE-Ras cells grown under non-permissive culture conditions. Colonies were stained with 0.005% w/v crystal violet in PBS containing 4% formaldehyde

Figure 9a illustrates the growth of tumours in BALB/c nude mice, wherein BALB/c female nude mice (8 week old) were injected subcutaneously with 0.2 mls of a single cell suspension containing 10^6 cells per site of injection. Tumour growth was assessed twice weekly. Neoplastic masses were measured using calipers. Tumour volume was calculated using the following formula: $V=\pi/6(\sqrt{(a.b)})^3$ [25]. The mean volumes and standard deviations of 4 tumours are represented, and

Figure 9b shows the growth in tumour size over time in animals injected with IMCE-Ras (clone 2) cell line in BALB/c nude mice.

Figure 9c shows the results of analysis of tumour tissue for Ras protein expression by means of the Ras autokinase assay. Lanes are as follows: (1) tumour tissue from IMCE-Ras cell line, (2) tumour tissue from YAMC-Ras cell line, and

Figure 9d shows the results of analysis of tumour samples for the presence of the mAPC gene.

Lanes are as follows:

(1) IMCE-Ras mAPC wild-type allele,

(2) IMCE-Ras mAPC mutant allele

(3) YAMC-Ras mAPC wild-type allele,

(4) YAMC-Ras mAPC mutant allele.

Example 1 Establishment of Conditionally-Immortalized Cell Lines

Heterozygous Immortomice (12) were purchased from Charles River Laboratories (Wilmington, MA.). The original Immortomouse strain was generated in CBA/Ca x C57B110 mice, and in the laboratory, the immortalizing gene is carried on a C57B16 background. Min/+ mice (1) were a gift from Dr Amy Moser, McArdle Laboratories, Madison, Wn. A heterozygous male Min mouse was mated with a female heterozygous Immortomouse, and the progeny were typed using PCR, as described below. Two of the 8 progeny were shown to carry both the Min mutation and the ts SV40 large T gene. The small intestine, colon and liver were removed from one of these two mice at 5 weeks of age and transported to the laboratory. Subsequently samples of other tissues were also taken.

In the same way, the following mouse crosses are performed:

Immortomouse x Myb transgenic (C57B16/SJL background)

Immortomouse x TGFα knockout transgenic (C57B16/OLA background)

Immortomouse x Ras transgenic (FVB background)

Matings of Immortomouse with the Ras transgenic mouse have been performed, and progeny have been born.

a) Small Intestinal and Colonic Cells

The small intestine and colon were opened and the contents removed by washing with saline. The culture technique has been described previously (11). In brief, the surface of these tissues was sterilised by soaking the tissues in 0.04% sodium hypochlorite in phosphate-buffered saline (PBS) for 20 minutes. The tissues were then washed in PBS and incubated for 90 minutes in a solution

containing 0.5mM EDTA and 0.05mM dithiothreitol. The incubating solution was then discarded and replaced with PBS, and the container shaken vigorously by hand for 1 minute. This shaking detached the crypts from the underlying stroma. The crypt suspension was placed in a centrifuge tube. Fresh PBS was added to the tube and the process repeated until the majority of the crypts had been released. The centrifuge tubes containing the crypt suspension were then centrifuged at 40g for 5 minutes. We have previously shown that when the colon is treated in this way, a suspension which contains only intact crypts and is free of fibroblasts and other stromal cells is obtained (10). When the small intestine is treated using this method, both crypts and villi are isolated. However, unlike the colonic crypt preparation, this preparation is not free of fibroblasts, as the villi contain stroma.

The crypt suspensions were cultured in RPMI 1640 medium containing 2.5% fetal calf serum, 10% conditioned medium from the human colonic epithelial carcinoma cell line LIM1863 (14) and 10 units per ml mouse γ-interferon (Boehringer Mannheim, Mannheim, Germany). The conditioned medium and γ-interferon are considered to be advantageous but not essential components of the medium. The cells were cultured at a concentration of 300 crypts per well in 24 well plates coated with rat tail collagen. The plates were incubated at the permissive temperature (33°C) in an atmosphere of 5% CO_2. Fresh γ-interferon was added to the wells every 48 hours for the first 10 days of the culture because the SV40 large T gene incorporated in these cells is coupled to a γ-interferon inducible promoter (12). A range of 1-100 units per ml is contemplated, but 10 units per ml is preferred. The medium was changed every 4 days until growth was established. When confluent, the cultures were split using trypsin/EDTA solution. Pure fibroblast cultures were obtained by trypsinization from the mixed epithelial-fibroblast cultures initially grown from the small intestinal crypts and villi. These

fibroblast cultures were grown in medium containing 10% fetal calf serum.

The crypts isolated from the colonic mucosa of the Immortomouse/Min hybrid mouse were cultured at the permissive temperature (33°C) in medium containing mouse γ-interferon. Many of the crypts attached to the collagen-coated plates within 24 hours. Unattached crypts were removed and replated into collagen-coated flasks. During the next 14 days the majority of the attached cells died; however, a few cells remained, and these cells proliferated and were passaged when sufficient cells were present. The cultured cells were stored in liquid N_2 before any assays were attempted. The Immortomouse/Min colonic epithelial (IMCE) cells grow as flat cuboidal cells, as shown in Figure 1. The morphology of the cells is similar to the immortomouse-derived colonic epithelial cell line young adult mouse colon (YAMC) described previously (11). Because the cultures were established from isolated crypts, no stromal cells were found in these cultures.

The crypt and villi mixture obtained from the small intestinal mucosa was also cultured. The villi are comprised of both epithelial and stromal cells. These cultures grew slowly, with small areas of epithelial cells being surrounded by a fibroblast monolayer. Pure fibroblast cultures (Immorto/Min fibroblasts; IMF) and pure epithelial cell cultures (Immorto/Min small intestine; ISI) have been isolated from these mixed cultures, and have been stored in liquid N_2.

b) <u>Liver cells</u>

To establish cultures from the liver, the tissue was minced with sharp scissors and then incubated in collagenase solution (100 units per ml; Collagenase IV, Sigma) for 60 minutes. The undigested tissue clumps were removed by filtration through a stainless steel mesh, and the small organoids and single cells were cultured in the

medium described above. To establish pure epithelial cultures a number of techniques were combined. Trypsin/EDTA was added to the mixed cultures of epithelial cells and fibroblasts, and the culture flasks were incubated until the majority of fibroblasts were released from the culture surface. This technique selectively increased the proportion of epithelial cells in the culture. In addition, the cells removed by trypsinization were replated into new flasks and incubated for 1 hour. After this time, the unattached cells were removed and transferred to a fresh flask. This process was repeated hourly for 4 hours. Using this selective adherence technique, it was found that the majority of the fibroblasts in the cell suspension adhered rapidly, with the epithelial cells being enriched in later platings. Finally, all cultures were grown in medium containing only 2.5% fetal calf serum.

c) <u>Other Cells</u>

Muscle and skin fibroblast cell lines were established using the same methods as for liver cells, and using tissue isolated from the same individual animal as before.

A gastric cell line, consisting of epithelial cells, has also been established in culture.

<u>Example 2</u> <u>Immunohistochemistry</u>

The colonic epithelial cells were cultured on Lab-Tek slide culture chambers (Nunc Inc, Naperville, Ill) at 33°C for 3 days. The cells were fixed in cold acetone, and the epithelial nature of the cultured cells was determined by staining with anti-keratin antibodies LE41 (keratin 8), LE61 (keratin 18) and LP2K, (keratin 19) kindly provided by Dr E.B. Lane, ICRF Laboratory, Dundee, Scotland (15). The cells were also stained using a polyclonal anti-keratin antibody which reacts with most keratin epitopes (Dako Corp., Carpinteria, CA), and were

tested for the presence of mucin using monoclonal antibodies specific for the Muc-1 and Muc-2 peptide repeat sequences (16). The cells cultured from the liver were also tested for the presence of keratin fibres using the same methods.

The cell monolayers stained strongly for keratin 18, with almost all cells showing characteristic keratin fibre staining (Figure 2), and less strongly for keratin 19, indicating the epithelial nature of these cells. The cells did not stain with the anti-keratin 8 monoclonal antibody. No Muc-1 or Muc-2 peptide staining was seen in these cultures.

The liver cultures initially contained a mixture of fibroblasts and epithelial cells. An Immorto/Min liver epithelial (IMLE) cell line was obtained by separating the epithelial cells from the stromal cells by differential trypsinisation and differential plating (Fig. 3). The cultured cells showed little staining with the antibody to keratin 18 but did stain with the polyclonal anti-keratin antiserum which recognises most keratin epitopes.

<u>Example 3</u> <u>Assays for Conditional Immortalisation</u>

The colonic epithelial cells were trypsinised and plated at $2x10^4$ per ml in the wells of 24-well dishes. The requirement of the cells for γ-interferon was tested by culturing in medium alone or medium containing 10 units/ml of γ-interferon. The conditional immortalization of the cells was tested by culturing duplicate plates at either the permissive temperature (33°C) or the non-permissive temperature (39.5°C) for 6 days. At the end of this incubation period, the cell proliferation in each well was assessed by trypsinizing the adherent cells and counting the total cell number per well. All assays were done in triplicate.

The fact that the IMCE cell line was only conditionally immortalized was demonstrated by the fact that there was no apparent growth, and considerable cell

loss, when the cells were cultured at the non-permissive temperature (39.5°C). In comparison, the cells grow rapidly at the permissive temperature (33°C). While γ-interferon was not essential, the growth of these cells was enhanced by the addition of mouse γ-interferon, further indicating the role of the SV40 large T protein in the growth of these cells. These results are illustrated in Figure 4.

Example 4 PCR Analysis

Mouse DNA was prepared from 1cm of tail tissue cut from mice greater than 2 weeks of age. In the case of cell lines, the cells were trypsinized and washed twice in PBS. DNA was isolated from both mouse tail tissue and cell lines as follows. Mouse tails or cell pellets were incubated in 750µl of homogenizing buffer (50mM Tris-HCl, pH 8; 100mM EDTA; 100mM NaCl; 1% SDS; 0.5mg/ml Proteinase K) overnight at 55°C. A volume of 310µl of 5M NaCl was added and the mixture centrifuged at 13,000rpm for 10 minutes. The supernatant was transferred to a new tube. Two/thirds of the supernatant volume of isopropanol was added and mixed for 2 minutes. The tube was then centrifuged at 13,000rpm for 1 minute and the pellet was washed with 70% ethanol and resuspended in 200µl of buffer (10mM Tris/HCl-1mM EDTA pH 8.0). Where necessary, DNA samples were stored at -70°C.

For each DNA sample two sets of PCR reactions were performed, one to detect the SV40 large T gene and the other to identify the Min mutant allele. The oligonucleotides used for testing the presence of the SV40 large T gene were

5'-CCTGGAATAGTCACCATG-3' (SEQ. ID NO: 1); and
5'-CAATGCCTGTTTCATGCC-3' (SEQ. ID NO: 2)

which respectively hybridize to nucleotides 3234 to 3251 and to nucleotides 2828 to 2845 of the SV40 genome, and

which each produce a 424bp PCR product. The oligonucleotides

5'-CTGAGAAAGACAGAAGTTA-3' (SEQ. ID NO: 3); and
5'-TTAGTGAAGTTGTATGTGTT-3' (SEQ. ID NO: 4) (17)

were used to test for the presence of the Min mutant allele; each results in a 280bp band.

Each PCR reaction contained 150ng DNA, 0.2mM each dNTP , 1.5mM $MgCl_2$ and 2.5U "Tth plus" DNA polymerase (Biotech International Ltd, Bentley, Western Australia). For the SV40 large T reaction, 6pM of each primer was used. For the Min reaction, 30pM of each primer was used. The reactions were overlaid with mineral oil and placed into a thermocycler, as described previously (17). At the completion of the PCR reaction, 16µl of the reaction mixture was electrophoresed on a 2% gel and the gel was photographed.

The results of the PCR assays for the presence of the Min mutation and SV40 large T gene in the cell lines cultured from the Immortomouse/Min hybrid are shown in Figure 5. It can be seen from lanes 4 and 11 that the colonic epithelial line (IMCE) carries both the Min mutation and SV40 large T gene, whereas the control colonic epithelial line (YAMC), derived from an Immortomouse, only expresses the SV40 large T gene (lanes 7 and 14). The liver cell line (IMLE) and the fibroblast line (IMF) derived from the hybrid mouse also carry both genes.

<u>Example 5</u> <u>Effect of the Gene of Interest on Temperature-Sensitivity</u>

Cells carrying the temperature-sensitive SV40 large T gene will grow only at the permissive temperature, 33°C, and will die if cultured at 37°C; the rate of cell death is even more rapid at 39.5°C than it is at 37°C. The ability of cell lines carrying this gene and a gene of interest to grow at 39.5°C is compared with the ability to

grow at 33°C, in order to determine whether the gene of interest has overcome the temperature-sensitivity of the cells. The ability to grow at the non-permissive temperature is the equivalent of the autonomous growth observed in tumour cells. This therefore provides a general test for identification of oncogenes, and tumour-inducing viruses.

The effect of oncogene-encoded proteins on the ability of conditionally-immortalized cell lines to grow autonomously was tested. In addition to growth at the non-permissive temperature, as described in the previous example, the ability to grow in the absence of γ-interferon was tested. These tests provide an assay for transformation of the cell lines.

YAMC and IMCE cells were infected with the viral supernatant from a Ψ2 cell line containing the activated v-H-ras gene (Ψ2 Ras), and then cloned in soft agar at the permissive culture conditions.

Infections were set up as follows: 0.5 ml of the supernatant from the Ψ2 Ras cell line was added to the target cells (5×10^3 cells per ml in a 5 cm petri dish) together with polybrene (SIGMA) at 4 μg/ml and incubated for 2 hours at 33°C. Following this incubation, the plates were flooded with 5 mls of complete medium [RPMI 1640 plus 10^{-5} M α-thioglycerol (ICN Chemicals), 1 unit/ml Insulin (SIGMA), 10^{-6} M Hydrocortisone (SIGMA)] plus 5 units/ml interferon-γ (murine recombinant GIBCO/BRL), and 10% v/v FCS. The cultures were incubated for a further 48 hours at 33°C in an atmosphere of 5% CO_2. The infected cells were then harvested and cloned in soft agar for selection at a density of 5×10^3 cells/plate. YAMC-Ras and IMCE-Ras cell lines were maintained in complete medium plus 5 units/ml interferon-γ, and 10% v/v FCS at 33°C in an atmosphere of 5% CO_2. After selection, clones were analysed for Ras protein expression by both a Ras autokinase assay and by Western blotting. The auto-kinase assay (22) was performed as follows: cell lysates were

prepared using LAU buffer (100 mM NaCl, 10mM Tris-HCl pH 7.5, 2mM EDTA, 0.5% sodium deoxycholate, 1% NP40, 10mM $MgCl_2$, 100 units trasylol, and 1 mM PMSF). After a 30 minute incubation on ice, lysates were spun at 12,000 rpm for 10 minutes. Ras protein was immunoprecipitated using the monoclonal antibody Y13-259 (Furth *et al*; J. Virol., 1982 <u>43</u> 294-304) and protein-G beads . The immunoprecipitated ras protein was then subjected to a kinase assay as follows: beads were washed once in LAU buffer and twice in TMD buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$ and 1 mM DTT). After the second wash protein-G beads were resuspended in 50 µl of TMD buffer plus 10mM [γ-^{32}P]GTP/5 mM cold ATP and incubated at 37°C for 30 minutes. The samples were then run on a 15% SDS/PAGE gel, proteins were transferred to immobilon PVDF membrane (Millipore) and phosphorylated bands were visualized by autoradiography. The blot was then subjected to Western blotting procedure using the Y13-259 antibody after which phosphorylated bands were visualised by ECL (Amersham). The mAPC genotype of the cell lines was determined as in Example 4. The results of analysis for genotype and for expression of Ras protein are shown in Figure 6.

Colony formation in soft agar was observed for both the YAMC and the IMCE cell lines under permissive and non-permissive conditions. Colonies were picked and cell lines expressing Ras , as determined by a Ras auto-kinase assay (22), were designated YAMC-Ras and IMCE-Ras.

Cells were seeded at 2,500 cells/well in 96 well plates in complete medium with or without interferon-γ at 5 units/ml and 1% FCS in a total volume of 200 µl/well. The cultures were then analysed each day for seven days. At each time point, 10 µl of MTT (5 mg/ml in PBS) was added and incubated a further 4 hours at the appropriate culture conditions. The culture medium was removed and the cells were solubilized with 200 µl of acid isopropanol (0.04 N HCl in isopropanol). The optical density was then

determined using a test wavelength of 560 nm and a reference wavelength of 690 nm [23]. Data representative of two independent experiments are shown in Figure 7.

At the permissive culture conditions all four cell lines (YAMC, YAMC-Ras, IMCE and IMCE-Ras) had similar doubling times (Figure 7a). The YAMC and IMCE cell lines did not proliferate when the immortalising conditions were shut down by culturing the cell lines at 39°C in the absence of interferon-γ (non-permissive culture conditions) (11, 23). However, the YAMC-Ras and IMCE-Ras cell lines continued to proliferate independently of the expression of the SV40 Large T antigen (Figure 7b). It is evident that in both the YAMC-Ras and IMCE-Ras cells the v-Ha-ras gene can replace the requirement for SV40 Large T antigen expression.

The morphology of the cell lines is illustrated in Figure 8a. That of the YAMC-Ras cell line was subtly changed compared to the parental YAMC cell line in that the cells were slightly refractile (compare Figure 8a i) and ii)). The IMCE-Ras cell line showed the typical spindle shaped appearance of a transformed cell (compare Figure 8a iii) and iv)). Furthermore, the IMCE-Ras cells were refractile, no longer contact-inhibited, and formed foci.

Soft agar colonies are illustrated in Figure 8b. Whilst the YAMC-Ras cells formed colonies in soft agar when incubated at the permissive culture conditions, they no longer did so when cultured in soft agar under the non-permissive culture conditions (compare Figure 8b ii) and iii) and Table 1 Column 1, 3). In contrast, the IMCE-Ras cells formed colonies in soft agar at both the permissive and non-permissive culture conditions (compare Figure 8b) v) and vi) and Table 1 Column 2, 4). Hence, the loss of one functional APC allele and expression of an activated ras gene were sufficient to transform mouse epithelial colonic cells.

Table 1

Colony Formation by Colonic Cell Lines in Soft Agar

Culture Conditions		Average No Colonies/Plate			
		33°C + γIF		39°C	
Cell Line		YAMC-Ras	IMCE-Ras	YAMC-Ras	IMCE-Ras
No Cells/ ml	10^4	39	>500	-	254
	10^3	4	159	-	71
	100	-	25	-	7
	50	-	6	-	1

Example 6 Ability of Conditionally-Immortalized Cell Lines to Induce Tumours

A variety of tumour cell lines, including human tumours, can be transmitted into the congenitally athymic nude (nu/nu) mouse.

The tumourigenicity of the YAMC-Ras and IMCE-Ras cell lines was assessed by injecting 0.2 ml of a single cell suspension containing 10^6 cells per site subcutaneously into nude mice. By 17 days all of the mice injected with IMCE-Ras cells had developed tumours with a mean volume of 650 ± 120 mm^3 (Figure 9a), which continued to express the activated Ras protein (Figure 9b)). At this time point there were no detectable tumours in the mice injected with the YAMC-Ras cells. However, these mice developed tumours by 90 days, with a mean volume of 970 ± 600 mm^3, which also continued to express the activated Ras protein (Figure 9b)). Neither of the parental cell lines induced tumours. Histological studies indicated that all tumours were adenocarcinomas.

The presence of the Min mutation in the cultured cells, as confirmed by the PCR studies, does not confer the property of autonomous growth on these cells. This is shown by the fact that the cells will not proliferate at

the non-permissive temperature (39.5°C), at which the SV40 large T protein is inactivated (13). The role of the SV40 large T protein in inducing growth in these cells was further demonstrated by the decrease in growth rate at the permissive temperature when γ-interferon was omitted from the medium. The fact that the IMCE cell line is only conditionally immortalized suggests that this cell line has retained the phenotype of normal intestinal mucosal cells, which cannot be cultured for long periods *in vitro*, especially if the cells are from adult animals (8,9).

These findings also indicate that the presence of the Min mutation alone is not sufficient to transform these cells. Although all the cells of the Min mouse carry the APC mutant gene, tumours have only been described in intestine and breast (1,20). Even in the intestine the majority of the intestinal mucosa remains histologically normal, with multiple focal tumours being formed as the animal ages. It has been shown that other mutations are required in order for malignant changes to occur. This is identical to the situation described in human colonic carcinoma by Vogelstein and colleagues, where multiple mutations are required before the malignant phenotype is expressed (18,21). The introduction of the Ras oncogene transformed the IMCE cell rapidly. Other genes known to be implicated in colon cancer, such as DCC, p53 or analogues thereof, should induce a malignant transformation in these cells, which in turn are expected to cause the cells to grow autonomously and thus to be able to grow at the non-permissive temperature. The IMCE cell line is a valuable tool for the study of these further events.

Other viruses or oncogenes which may also transform the cell line include, but are not limited to, herpes, Epstein-Barr virus, papillamovirus, myc and raf.

Thus, it is possible to derive "normal" epithelial cell lines using the present invention and compare such cells to their transformed equivalents.

Thus the invention makes it possible to study the

stages leading to malignant transformation by introducing genetic changes into the normal cells and observing the progression through to tumourigenesis of such cells. This is of importance in humans, as the vast majority of human cancers develop in proliferating, less differentiated cells particularly in epithelial tissues (adenocarcinomas).

Another interesting observation in the Min mouse system is the recent description of a modifier locus that seems to govern the development of tumours in F1 hybrids of Min mice with AKR/J and MA/Myj mouse strains (2,4). The hybrid from which the cells of this invention were derived was on a C57Bl/6 background, so that these modifier loci should not apply to the cells of the present invention.

The Min mouse has also been found to develop mammary tumours (20), but no hepatic tumours have been described in these mice. The availability of cell lines derived from liver and stroma from the same mouse allows the study of the tissue specific elements that lead to these mice developing small intestinal and colonic tumours but not liver cancers. Similarly, fibroblast and epithelial cell lines from the same animal can be used to elucidate the role of cell type in cancer development.

Our results also demonstrate the usefulness of the Immortomouse and similar transgenic mice as a means of introducing an immortalizing gene into any other transgenic mouse strain that expresses a gene of interest. Once the SV40 large T gene is present in the cells it will be possible to culture a range of cell types that have previously not been grown in long term culture (24-26).

The ability to transform the immortalised cell line and render it tumourigenic as measured by the ability to grow as a culture under both permissive or non-permissive conditions also provides a rapid assay for tumourigenic properties.

It will be apparent to the person skilled in the art that while the invention has been described in some

detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

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CLAIMS

1. An F1 hybrid non-human animal, of which one parent carries an immortalizing gene, and the other parent carries one or more genes of interest.

2. An F1 hybrid non-human animal according to Claim 1, wherein the immortalizing gene is a gene that confers the capacity for long term growth in cell culture.

3. An F1 hybrid non-human animal according to Claim 1 or Claim 2, wherein the immortalizing gene is selected from the group consisting of the SV40 large T gene, the adenovirus EIA gene and the polyoma virus middle T gene.

4. An F1 hybrid non-human animal according to Claim 1 or Claim 2, wherein the immortalizing gene is a combination of genes selected from the group consisting of the SV40 large T gene, the adenovirus EIA gene and the polyoma virus middle T gene.

5. An F1 hybrid non-human animal according to any one of Claims 1 to 4, wherein the immortalizing gene is a temperature sensitive mutant of the SV40 large T gene.

6. An F1 hybrid non-human animal according to Claim 5, wherein the parent carrying the immortalizing gene is the Immortomouse (H-2k^b-tsA58 SV40 large T).

7. An F1 hybrid non-human animal according to any one of Claims 1 to 6, wherein the gene of interest is a gene for which a transgenic, non-human animal species can be generated.

8. An F1 hybrid non-human animal according to any one of Claims 1 to 7, wherein the gene of interest is one or more genes selected from the group consisting of a cancer-associated gene, an oncogene, a tumour-suppression gene and a tumour inhibitor gene.

9. An F1 hybrid non-human animal according to any one of Claims 1 to 8, wherein the gene of interest predisposes towards development of cancer.

10. An F1 hybrid non-human animal according to any one of Claims 1 to 9, wherein the gene of interest bears

one or more mutations.

11. An F1 hybrid non-human animal according to any one of Claims 1 to 10, wherein the gene of interest is a transgene.

12. An F1 hybrid non-human animal according to any one of Claims 1 to 11, wherein the gene of interest is one or more genes selected from the group consisting of Multiple Intestinal Neoplasia (Min) or homologues or derivatives thereof, Adenomatous Polyposis Coli (APC), Deleted in Colon Carcinoma (DCC), Ras, Myb, Myc, Raf, p53 and p16.

13. An F1 hybrid non-human animal according to Claim 12, wherein the gene of interest is the Multiple Intestinal Neoplasia gene, or a homologues or derivative thereof.

14. An F1 hybrid non-human animal according to any one of Claims 1 to 6, wherein the gene of interest is a cancer-associated gene or an oncogene.

15. An F1 hybrid non-human animal according to any one of Claims 1 to 6, wherein the gene of interest is one or more genes selectively disrupted to prevent expression thereof.

16. An F1 hybrid non-human animal according to Claim 15, wherein the disrupted gene of interest is selected from the group consisting of a gene encoding a cytokine, growth factor, hormone and enzyme.

17. An F1 hybrid non-human animal, of which one parent carries a temperature-sensitive mutant of the SV40 large T gene, and the other parent carries the Multiple Intestinal Neoplasia gene.

18. An F1 hybrid non-human animal according to any one of Claims 1 to 17, wherein the animal is selected from the group consisting of rabbit, mouse and rat.

19. An immortalized or conditionally-immortalized cell line, the cells of which carry an immortalizing gene and one or more genes of interest.

20. An immortalized cell line derived from an animal according to any one of Claims 1 to 18.

21. An immortalized cell line according to Claims 19 or 20, wherein the immortalized cell line is selected from the group consisting of colonic epithelial cells, liver epithelial cells, small intestinal epithelial cells, small intestinal fibroblasts, muscle fibroblasts and skin fibroblast.

22. An immortalized cell line according to Claim 21, wherein the cells are derived from an F1 hybrid non-human animal, of which one parent carries a temperature-sensitive mutant of the SV40 large T gene and the other parent carries the Multiple Intestinal Neoplasia gene.

23. An immortalized cell line according to Claim 22, wherein the cell line is transfected with further genes of interest, said genes being selected from the group consisting of Ras, Myb, Myc, Raf, p53 and p16.

24. An immortalized cell line according to Claim 23, wherein the cell is transfected with the Ras oncogene.

25. A method of obtaining an immortalized or conditionally immortalized cell line, the cells of which carry an immortalizing gene and one or more genes of interest, comprising the step of:

a) crossing a non-human animal bearing an immortalizing gene with an animal of the same species bearing the gene of interest,

b) obtaining an F1 hybrid animal, and

c) culturing cells from the F1 hybrid animal under conditions suitable for growth thereof.

26. A method according to Claim 25, wherein a mouse carrying one or more genes of interest is crossed with a mouse carrying a temperature sensitive mutant of SV40 large T gene to obtain an F1 hybrid mouse, and culturing cells from the F1 hybrid mouse at a permissive temperature of 33°C.

27. A method according to Claims 25 or 26, wherein the mouse carrying the immortalized gene is the

Immortomouse (H-2k^{b}-tsA58 SV40 large T) and the mouse carrying the gene of interest is the Multiple Intestinal Neoplasia mouse.

28. The use of an immortalized or conditionally immortalized cell line carrying one or more genes of interest to screen for agents selected from the group consisting of carcinogenic agents, anti-carcinogenic agents, anti-mitotic agents, mutagens and antibodies.

29. The use of an immortalized cell line according to Claim 28, wherein the agent being screened consists of DNA from tumours or tumour homogenates.

30. Use according to Claim 28, wherein the agent is selected from the group consisting of therapeutic agents, immunological response modifiers, receptors, hormones, antibodies, cytokines, and growth factors.

31. Use according to any one of Claims 28 to 30, wherein the gene of interest is Ras.

32. Use according to any one of Claims 28 to 30, wherein the cell line induces tumours when injected into nude mice.

AMENDED CLAIMS

[received by the International Bureau on 5 December 1995 (05.12.95); original claims 1-32 replaced by amended claims 1-25 (4 pages)]

1. A transgenic non-human animal having integrated into its genome an immortalizing gene and a multiple intestinal neoplasia-associated gene or a homologue or derivative thereof, wherein expression of said multiple intestinal neoplasia-associated gene or homologue or derivative thereof is controlled by said immortalizing gene.

2. The animal of claim 1 wherein said immortalizing gene confers the capacity for long term growth in cell culture.

3. The animal of claim 1 wherein said immortalizing gene is selected from the group consisting of SV40 large T gene, adenovirus EIA gene, polyomavirus middle T gene and combinations thereof.

4. The animal of claim 3 wherein said immortalizing gene is a temperature-sensitive mutant of SV40 large T gene.

5. The animal of claim 1 wherein said multiple intestinal neoplasia-associated gene or homologue or derivative thereof predisposes toward cancer development.

6. The animal of claim 1 wherein said multiple intestinal neoplasia-associated gene or homologue or derivative thereof bears at least one mutation.

7. The animal of claim 1 wherein said multiple intestinal neoplasia-associated gene or homologue or derivative thereof is selectively disrupted to prevent expression thereof.

8. The animal of claim 7 wherein said multiple intestinal neoplasia-associated gene or homologue or derivative thereof is selected from the group consisting of a gene encoding a cytokine, a gene encoding a growth factor, a gene encoding a hormone and a gene encoding an enzyme.

9. The animal of claim 1 wherein said animal is selected from the group consisting of a rabbit, a mouse and a rat.

10. A cell line which carries an immortalizing gene and a multiple intestinal neoplasia-associated gene or homologue or derivative thereof.

11. The cell line of claim 10 wherein said immortalizing gene confers the capacity for long term growth in cell culture.

12. The cell line of claim 10 wherein said immortalizing gene is selected from the group consisting of SV40 large T gene, adenovirus EIA gene, polyomavirus middle T gene and combinations thereof.

13. The cell line of claim 12 wherein said immortalizing gene is a temperature-sensitive mutant of SV40 large T gene.

AMENDED SHEET (ARTICLE 19)

14. The cell line of claim 10 wherein said multiple intestinal neoplasia-associated gene or homologue or derivative thereof predisposes toward cancer development.

15. The cell line of claim 10 wherein said multiple intestinal neoplasia-associated gene or homologue or derivative thereof bears at least one mutation.

16. The cell line of claim 10 wherein said multiple intestinal neoplasia-associated gene or homologue or derivative thereof is selectively disrupted to prevent expression thereof.

17. The cell line of claim 16 wherein said multiple intestinal neoplasia-associated gene or homologue or derivative thereof is selected from the group consisting of a gene encoding a cytokine, a gene encoding a growth factor, a gene encoding a hormone and a gene encoding an enzyme.

18. The cell line of claim 10, wherein said cell line is derived from a rabbit, a mouse or a rat.

19. The cell line of claim 10, wherein said cell line is selected from the group consisting of colonic epithelial cells, liver epithelial cells, small intestinal epithelial cells, small intestinal fibroblasts, muscle fibroblasts and skin fibroblasts.

20. The cell line of claim 10, wherein said cell line is also transfected with a gene of interest, said gene of interest being

selected from the group consisting of Ras, Myb, Myc, Raf, p53 and p16.

21. The cell line of claim 20, wherein said cell line is transfected with Ras oncogene.

22. A method of obtaining an immortalized or conditionally immortalized cell line, the cells of which carry an immortalizing gene and a multiple intestinal neoplasia-associated gene or homologue or derivative thereof, comprising:

a) crossing a non-human animal carrying an immortalizing gene with an animal of the same species carrying a multiple intestinal neoplasia-associated gene or homologue or derivative thereof so as to obtain a hybrid animal; and

b) culturing cells from said hybrid animal under conditions suitable for growth thereof.

23. The method of claim 22, wherein said animal carrying a multiple intestinal neoplasia-associated gene or homologue or derivative thereof is crossed with an animal carrying a temperature sensitive mutant of SV40 large T gene.

24. The method of claim 22 wherein said animal is a mouse.

25. The method of claim 24, wherein the mouse carrying the immortalized gene is the Immortomouse (H-2k^b-tsA58 SV40 large T).

AMENDED SHEET (ARTICLE 19)

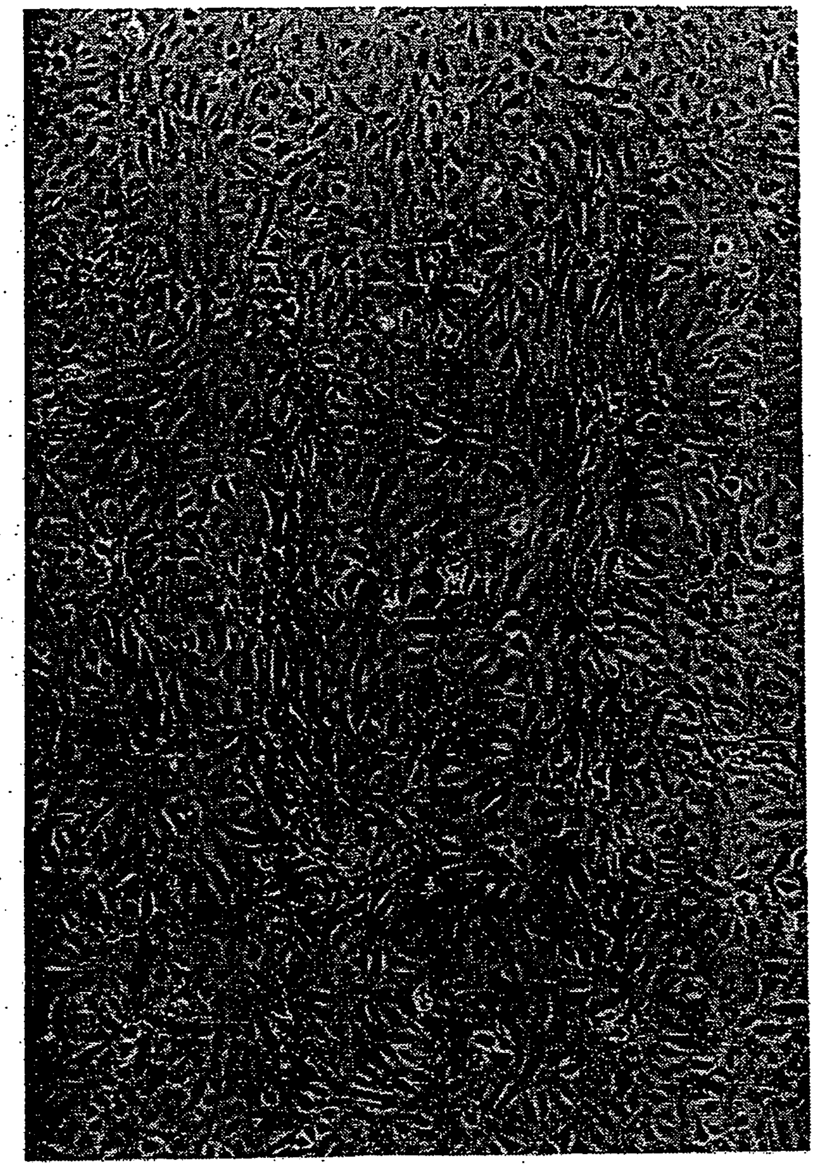


FIG. 1

FIG. 2

SUBSTITUTE SHEET (RULE 26)

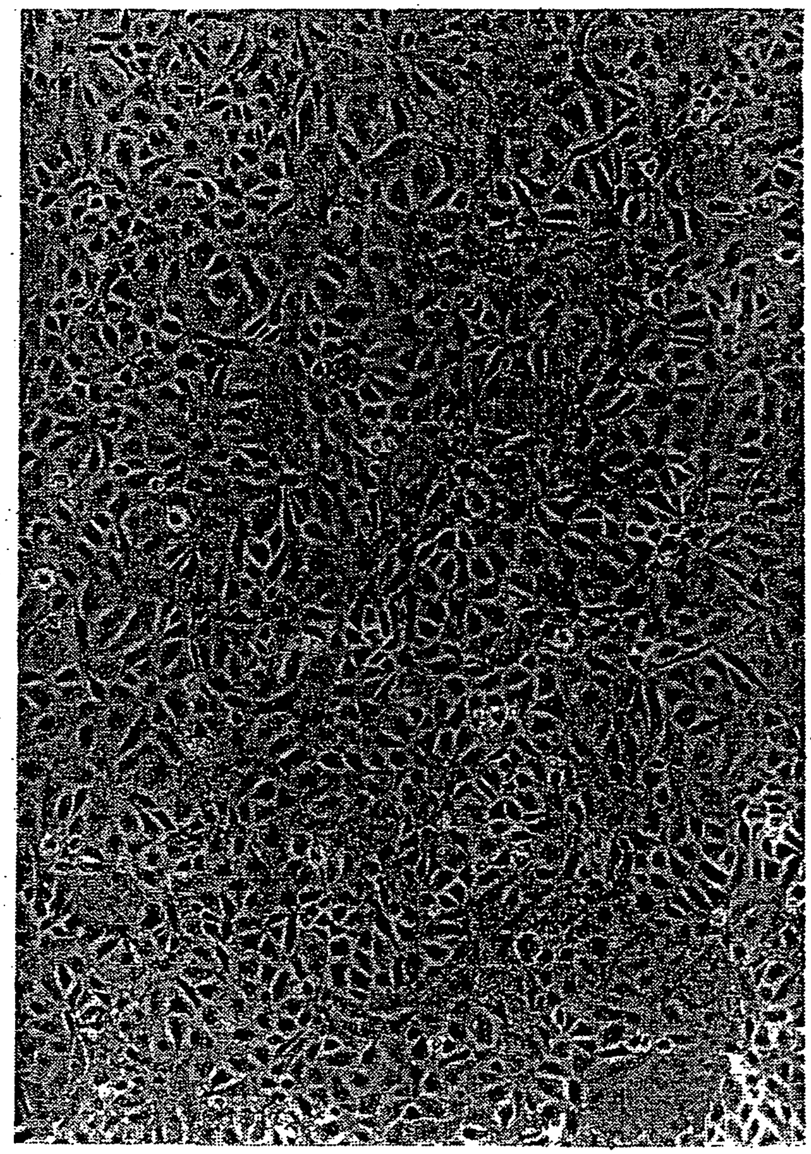


FIG. 3

FIG. 4

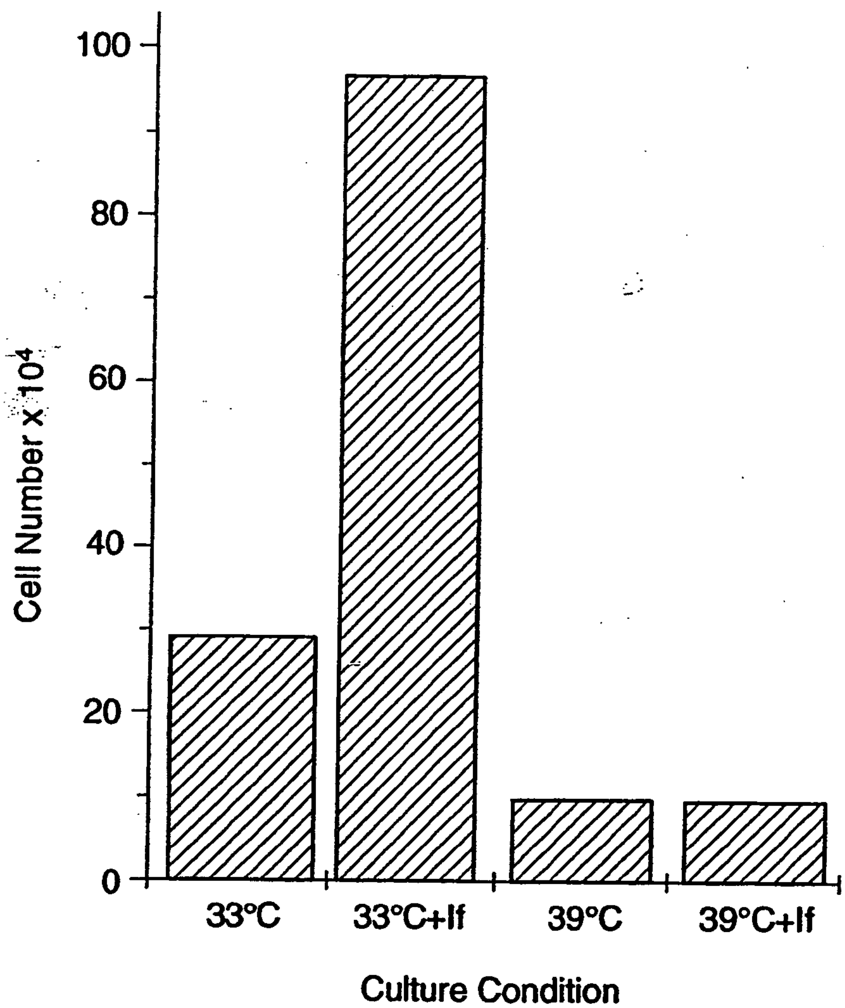

100
80
60
40
20
0
Cell Number x 10^4
33°C
33°C+If
39°C
39°C+If
Culture Condition

FIG. 5

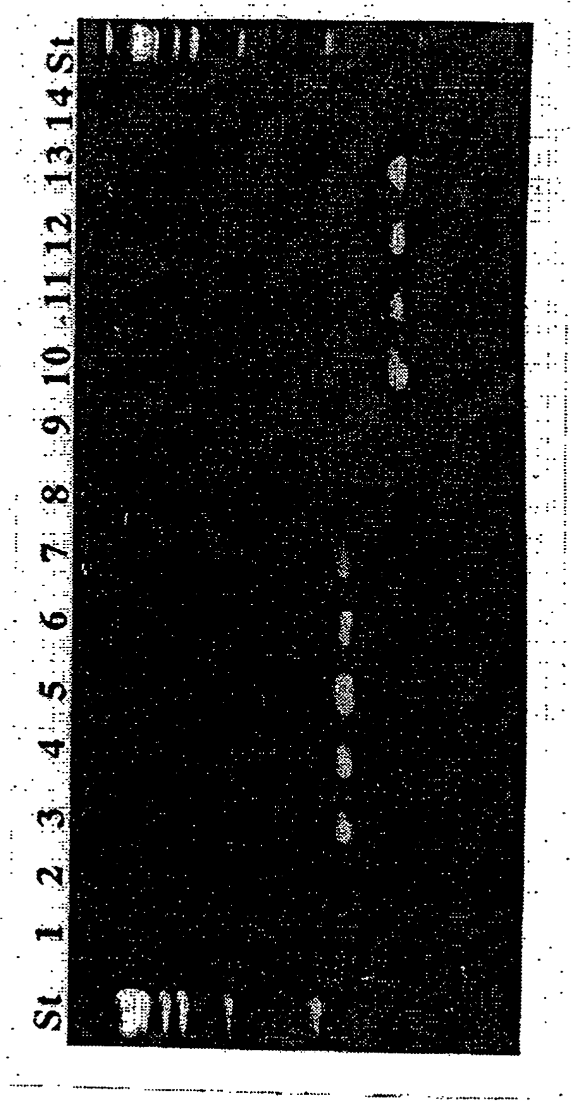


FIG. 6

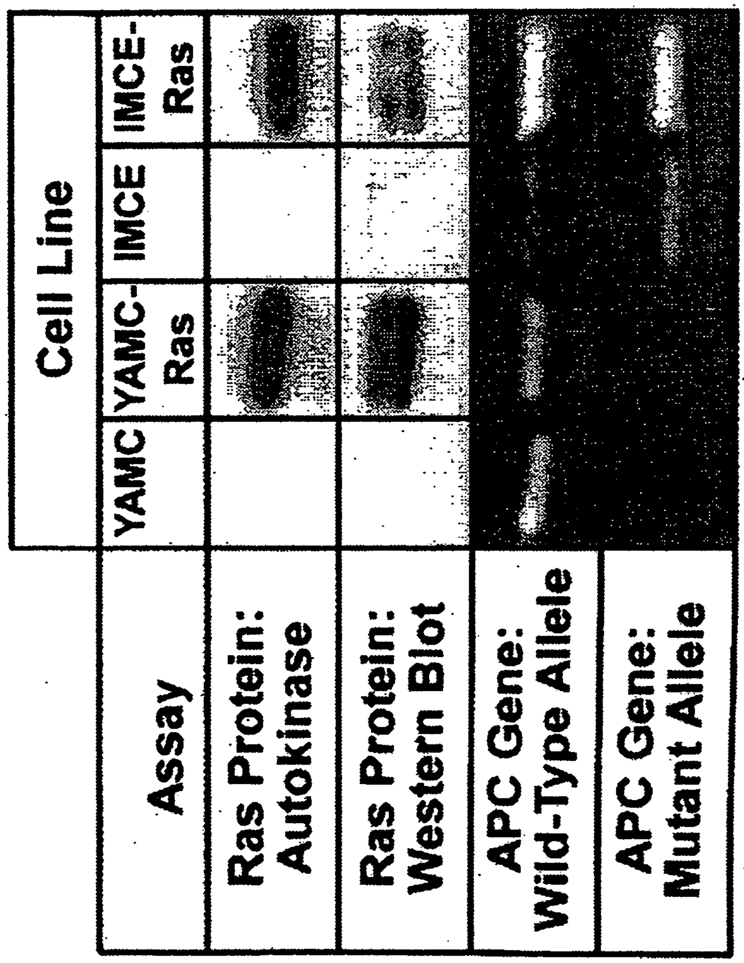

Cell Line
YAMC
YAMC-Ras
IMCE
IMCE-Ras
Assay
Ras Protein: Autokinase
Ras Protein: Western Blot
APC Gene: Wild-Type Allele
APC Gene: Mutant Allele

FIG. 7a

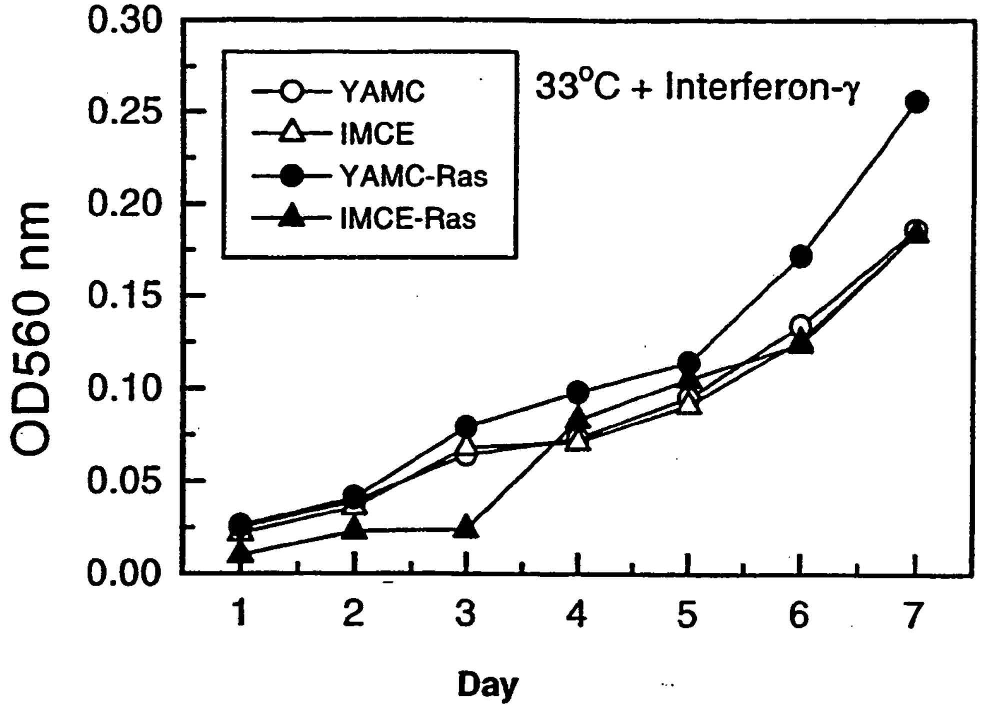

0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD560 nm
YAMC
IMCE
YAMC-Ras
IMCE-Ras
33°C + Interferon-γ
1
2
3
4
5
6
7
Day

FIG. 7b

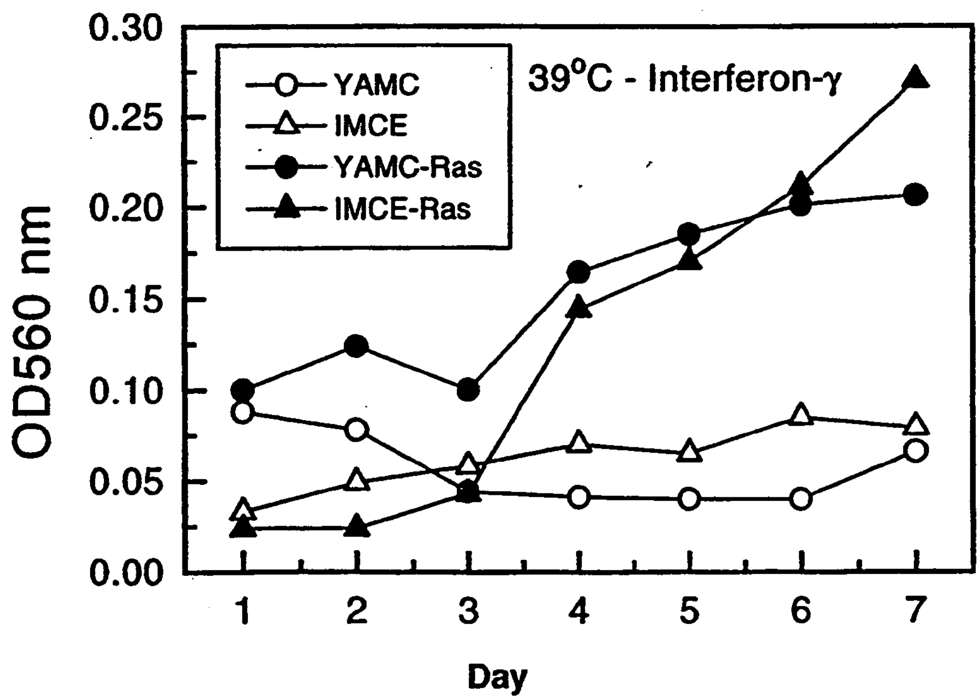

0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD560 nm
YAMC
IMCE
YAMC-Ras
IMCE-Ras
39°C - Interferon-γ
1
2
3
4
5
6
7
Day

FIG. 8a

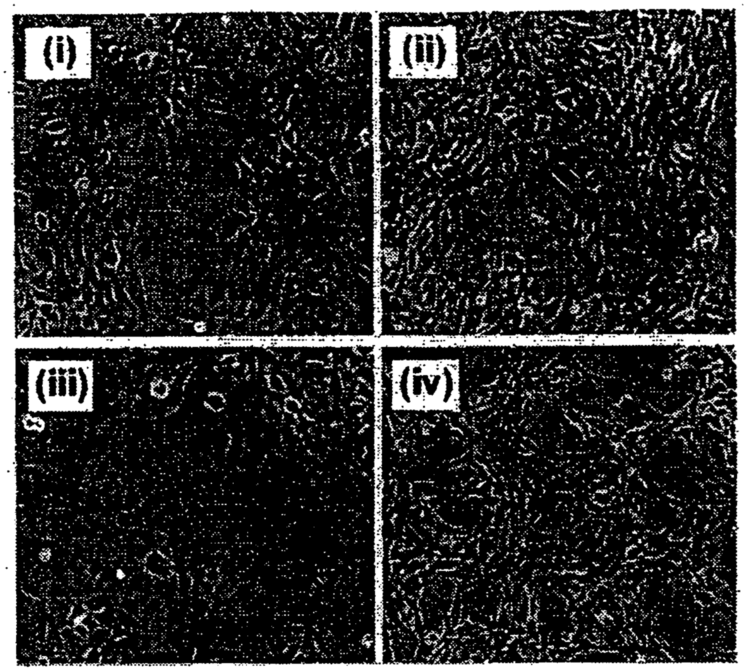


FIG. 8b

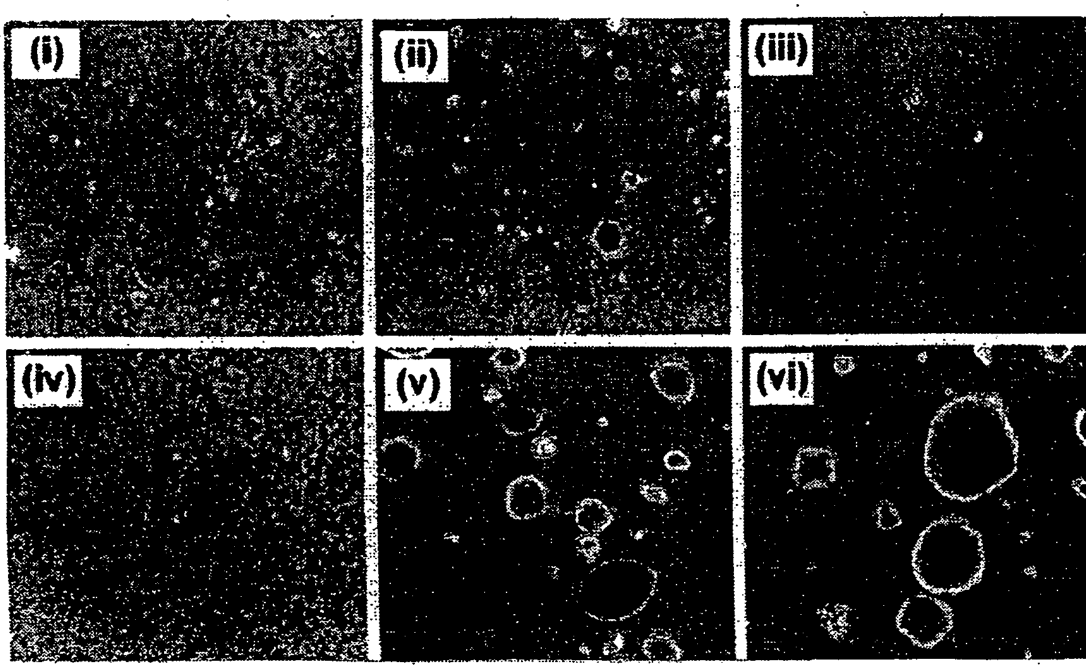


FIG. 9a

Clone	Tumour Incidence	Latency Time Days	Tumour Volume mm³ ±SEM
IMCE-Ras.1	4/4	21	770 ± 360
IMCE-Ras.2	4/4	17	650 ± 120

FIG. 9b

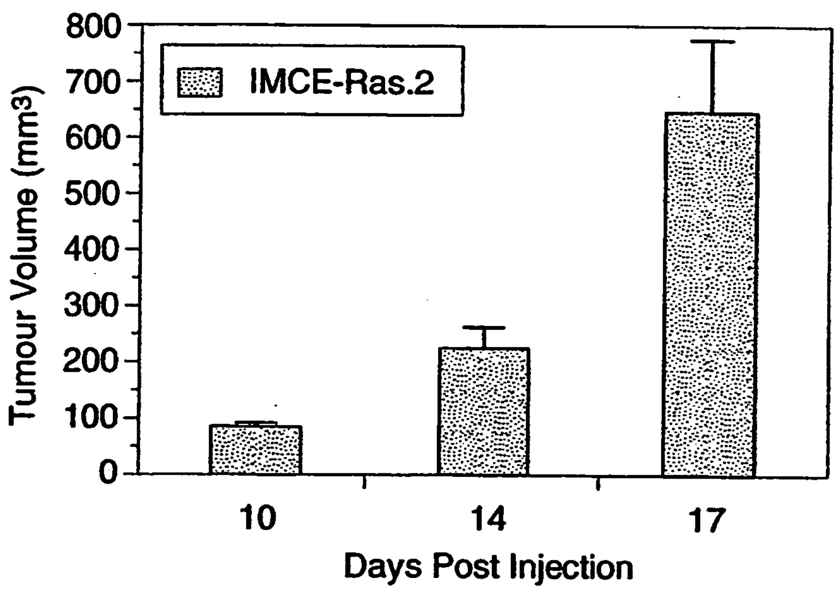


FIG. 9c

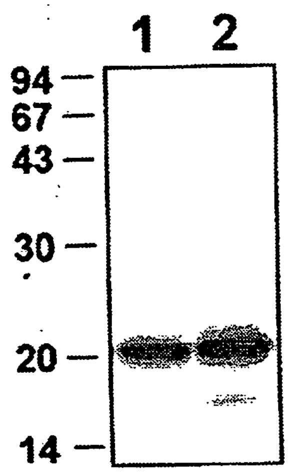
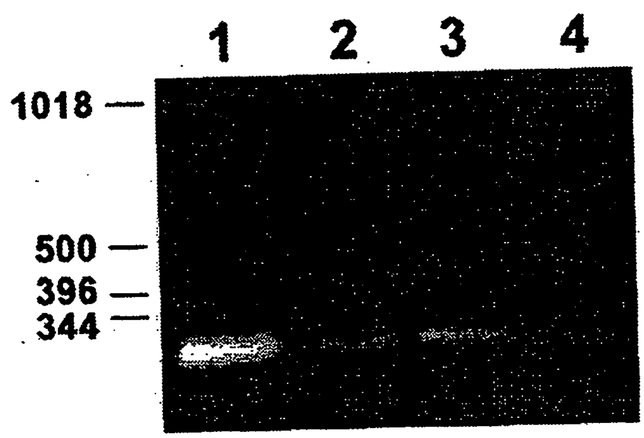


FIG. 9d



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07255

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12N 15/00, 5/00
US CL : 800/2; 435/240.2, 172.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 800/2, DIG 1; 435/240.2, 172.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, DIALOG (files 154, 55)
search terms: Min, Apc, SV40, transgenic, immortal, cross, culture, tumor, inventors' names

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P ---- Y	Epith. Cell Biol., Volume 3, issued 1994, Whitehead et al., "Derivation of conditionally immortalized cell lines containing the Min mutation from the normal colonic mucosa and other tissues of an "Immortomouse"/Min hybrid", pages 119-125, see the entire document.	1-27 ------- 28-32
Y	Proc. Natl. Acad. Sci. USA, Volume 88, issued June 1991, Jat et al., "Direct derivation of conditionally immortal cell lines from an H-2Kb-tsA58 transgenic mouse", pages 5096-5100, see the entire document.	1-32

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 SEPTEMBER 1995	05 OCT 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Della Collins for JASEMINE C. CHAMBERS Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07255

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proc. Natl. Acad. Sci. USA, Volume 90, issued January 1993, Whitehead et al., "Establishment of conditionally immortalized epithelial cell lines from both colon and small intestine of adult H-2Kb-tsA58 transgenic mouse", pages 587-591, see the entire document.	1-32
Y	Science, Volume 256, issued 01 May 1992, Su et al., "Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene", pages 668-670, see the entire document.	1-32
Y	Genomics, Volume 15, issued 1993, Luongo et al., "Mapping of multiple intestinal neoplasia (Min) to proximal chromosome 18 of the mouse", pages 3-8, see the entire document.	1-32
Y	Cell, Volume 75, issued 19 November 1993, Dietrich et al., "Genetic identification of Mom-1, a major modifier locus affecting Min-induced intestinal neoplasia in the mouse", pages 631-639, see the entire document.	1-32

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